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THE STABILITY AND SOLUBILITY OF DIPHTHERIA TOXIN IN ACID AND ALKALI.

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THERE are observations from 1890 onwards that diphtheria toxin when acidified loses its toxicity, which is partially recovered on reneutralisation (Doerr 1908). The fact that this loss is due to precipitation was overlooked until Glenny and Walpole (1915) made it the basis of a method for purifying toxin. Alkali has on the whole been found destructive and its effect irreversible. Walbum (1922) showed that between *p*H 9·5 and 10·5 destruction was complete after 6 days at 37° C. The aim of the present experiments was to determine the stability, as distinct from the precipitation, of toxin with acid and alkali in terms of *p*H, and incidentally the frequent incompleteness of precipitation with acid alone led to the trial of other precipitants.

Already in 1890 Frankel and Brieger had found that precipitation of toxin with alcohol was most complete if the alcohol was slightly acidified with acetic acid. This precipitant is most effective at that pH where the solubility of the toxic material is already at a minimum; and, since it is not completely insoluble at the isoelectric point, an additional precipitant is needed.

Alum was chosen for the following experiments as it had already been shown to give highly antigenic precipitates (Glenny, Pope, Waddington and Wallace 1926). Ammonium sulphate was used by Brieger and Boer in 1896, but the toxicity of ammonium salts is a disadvantage in the purification of diphtheria toxin for immunising purposes.

All such methods have one disadvantage; the precipitation of the toxin depends upon, or is inseparable from, the precipitation of other material already present in the culture filtrate. It has been shown that this acid precipitable material is often present in the original medium, that it increases during the growth of *C. diphtheriæ* and that its increase bears no relation to the strength of toxin obtained (Watson and Wallace 1924*a*). Consequently both the purification (toxin units per mgrm. of total weight) and the yield of toxin obtainable by precipitation is liable to vary for different toxins.

Adsorption on to some material not naturally present in the culture media, or formed during growth, would give a less variable method of purification.

Outline of experiments.

In a previous publication it was shown that the loss in yield of toxin throughout a series of toxins precipitated with acid was never less than 40 per cent. (Watson and Wallace 1924*b*). This may have been due to any or all of the following causes:—destruction; loss in handling the slight precipitate; incomplete precipitation of the toxins; under-estimation of the purified toxin by measuring the Lr/500 dose, since the toxins may have been partially "toxoided" during the process (Doerr 1908). These points have now been investigated separately. The destruction is traced from *p*H 0·5 to *p*H 11·0 in a series of controls in which loss in handling the precipitate is eliminated; the partition of the toxin between the clear solution and the precipitate is estimated over the three separate ranges of *p*H where precipitation occurs; the value of the toxin is determined by Ramon's flocculation method which measures the sum of the toxin and toxoid present.

Samples of the toxin were brought to various hydrogen ion concentrations with HCl and NaOH of such strengths as to make the change in volume negligible in the estimation of the toxins *i.e.* the maximum increase was 5 per cent. After two days at room temperature, the limits of precipitation were noted: the whole of the clear samples, and one half of those which had precipitated, were then readjusted to the *p*H of the original toxin. The remaining half of each of the precipitated samples was centrifuged: the supernatants separated, and readjusted to *p*H 8·0 after filtering. The precipitates were washed with the original volume of diluted buffer solution of the same *p*H as the solution from which they were separated. They were finally dissolved with a trace of NaOH and diluted to the original volume at *p*H 8·0. This routine was first carried out with the original toxin containing 0·2 per cent. formalin, then with the same toxin to which 0·5 per cent. phenol had been added. Later, with the object of a more complete precipitation of the toxin, 2 per cent. alum was added, and to investigate the action of the alum it was added in the presence of tartrate (charts 1-4).

Measurement of the toxin.

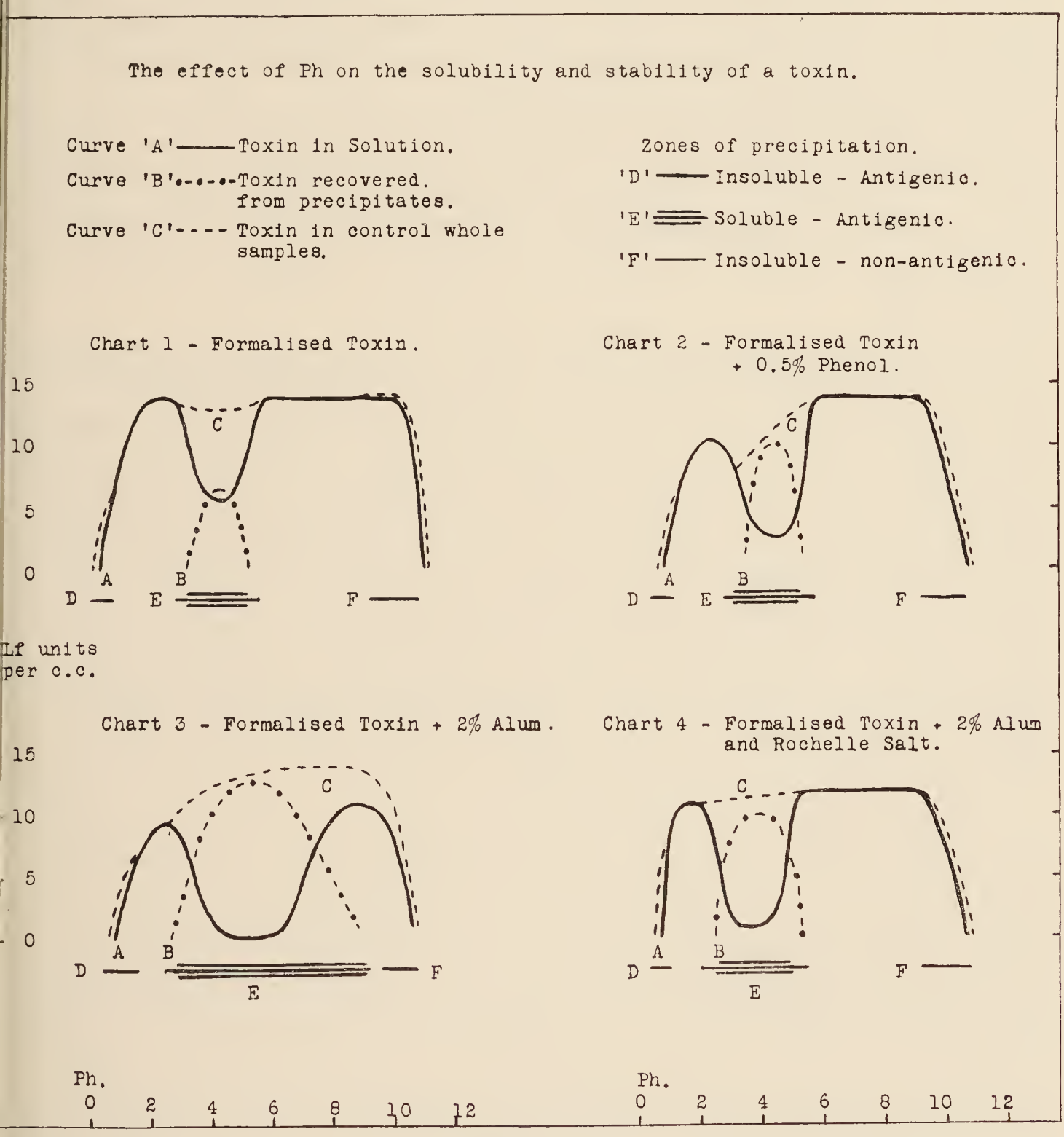
Toxin is characterised by three different effects: toxicity, combination with antitoxin and antigenic power; each has given rise to separate methods of measurement and until all three effects have disappeared the toxin cannot be said to have been destroyed. The original toxin was already partially toxoided, therefore no attempt was made to measure further changes by toxicity. Combination with antitoxin was measured in terms of Lf units per c.c. by titration with standard serum as described by Glenny and Wallace (1925), and antigenic values by the comparative method of the immunity index (Glenny, Allen and Hopkins, 1923).

The flocculation test may give erroneous results when applied to toxins concentrated by acid precipitation. Other substances occurring in the culture filtrate may be simultaneously concentrated and give non-specific zone flocculations. Such flocculations may or may not appear with normal horse serum and can only be distinguished by titrating against several different standard sera, when the non-specific flocculation, should it again appear, will be seen to bear no constant relation to the antitoxic content of the various sera. One such instance has been published by Glenny and Wallace (1925), and since several others have subsequently occurred it is advisable to check the Lf value of treated toxins by some animal test. Toxins which have been treated with acid or alkali may give no flocculation with antitoxin. It does not follow that they have been destroyed, for their Lf value may still be measured by blending with fresh toxin. In these experiments the original toxin was

used throughout as the blend toxin; it contained 14 Lf units per c.c. and flocculated in thirty minutes at 50° C. It was itself titrated to 8 per cent. The standard and unknown toxins were mixed in equal volumes, so that the minimum Lf value detectable in the unknown toxin was 1 Lf unit per c.c. The accuracy of the titrations therefore decreased proportionally from 10 per cent. for a toxin containing 14 units to 100 per cent. to 200 per cent. for a value of 1 unit. All the points below two units in charts 1-4 are therefore only very approximate.

Measurement of reaction.

The *p*H of the solutions in the earlier experiments (chart 1) were measured colorimetrically by comparison with standard buffer solutions. This was not



CHARTS 1-4

convenient through lack of suitable indicators for the extreme acid and alkaline ranges. In the later experiments the *p*H was measured electrometrically using a quinhydrone electrode (Pope and Gowlett, *in press*) up to *p*H 8·0 and a glass electrode for the more alkaline solutions. The samples were tested within 1 to 2 hours after the addition of acid and alkali and any later change during the 2 days' standing was not taken into account.

The effect of acid and alkali on formalised toxin. Chart 1.

The initial *p*H of the toxin was 7·2. Various amounts of HCl and NaOH were added to separate samples which were kept at room temperature; 48 hours later all those less than *p*H 1·0, greater than *p*H 9·0, and between *p*H 2·5 and 5·5 had precipitated. The precipitate was heaviest and appeared first round *p*H 4·0 in the middle of this zone. From curve A which represents the amount of toxin in solution as measured by Lf value at any *p*H, it will be seen that there is no change in Lf units per c.c. as the *p*H falls from 8 to 6. At *p*H 5·0 with the first trace of precipitate the Lf value falls and reaches a minimum of 6 units at *p*H 4·4. It then gradually rises as the precipitate redissolves until at *p*H 2·4 the solution is clear and the original value of 14 Lf units is reached.

At *p*H 1·0, when the second precipitation appears, the Lf value again falls and no Lf units are detectable at *p*H 0·4. On the alkaline side a slight precipitate appears at *p*H 9·0 with no loss of toxin, but between *p*H 9·6 and 11, the Lf value falls abruptly from 14 to less than 1 unit.

Curve B, representing the amount of toxin precipitated at any *p*H, shows that the Lf units which disappear from the clear solution (curve A) at *p*H 5·0 are recoverable from the precipitate when it is redissolved, so that the sum of the ordinates of curves A and B represents the total toxin. The precipitates in this acid zone *p*H 5·0 to 2·5, and the whole suspensions readjusted without separating the precipitate, (curve C) were completely soluble in a trace of alkali, and within the limits of experimental error all the toxin was recoverable. This sample of toxin concentrated by acid precipitation at the optimum *p*H gives a yield of only 50 per cent. The loss is not due to destruction but to incomplete separation of the toxin, more than half still remaining in solution.

In the excess acid precipitation zone, *p*H 1·0 and less, the toxin disappearing from the filtrates was not recoverable from the precipitates. These precipitates did not dissolve on re-adjusting the *p*H with alkali, either when suspended in water or in supernatant as in the control series; the curves for the toxin in the control series and in the filtrates here coincide. The alkaline precipitates were also insoluble in neutral solution.

Excess acid, and alkali appear to have destroyed the toxin but the flocculation method only measures soluble toxin. The stability of the toxin as an antigen at several points in the preceding curves was therefore also determined by injecting the neutralised samples into guinea-pigs. At *p*H 3·5 and 4·2, when the Lf units are divided between the precipitate and filtrate, both fractions are antigenic but neither equal to the original. The fractions precipitated with HCl between *p*H 1·0 to 0·6 were approximately equal in antigenic value to the original toxoid, and the filtrates still slightly antigenic. Nitric

acid also precipitates the antigen unharmed at *p*H 0·2. An injection of 5 c.c. of toxin which had been kept at this *p*H for 3 days raised the antitoxic titre of a rabbit from one-fifth to twenty units of antitoxin per c.c. within 7 days. There was on the other hand only a slight immunity response to the precipitates separated from the alkaline solutions. The antigenic power of the control suspensions diminished at *p*H 10 and little remained at *p*H 11.

The antigenic value of the insoluble precipitates below *p*H 1 would indicate that the precipitated toxin was insoluble *in vitro* and soluble *in vivo*. Unless the antigenic efficiency of the insoluble precipitate was far greater, unit for unit, than that of the original toxin, no destruction can have occurred. It was found later that if the toxin was kept at *p*H 1 or less for a shorter time, 2 hours at room temperature, the precipitate was soluble on neutralisation and this solution was approximately of the same strength as the original toxin. So long therefore as the precipitates do not become insoluble there appears to be little destruction caused by acid.

Modification of toxin.

Maloney and Weld (1925) found that formaldehyde modifies toxin into toxoid more rapidly in alkaline than in acid solutions. We have confirmed this observation which must be taken into account in considering the toxoiding action directly due to the *p*H of this toxin to which 0·2 formaldehyde had been added. The original toxin contained 14 Lf units and 500 M.R.D. per c.c. At *p*H 3·4 the filtrate had 10 Lf units and only 10 M.R.D. per c.c., showing that considerable modification had occurred; while the redissolved precipitate contained 2·5 Lf units and 100 M.R.D., showing little or no modification. If there was no separation of toxic and binding units by the precipitation with acid as shown by Glenny and Walpole (1915), the precipitated toxin must be less rapidly modified by acid than toxin in solution.

The effect of acid and alkali on formalised toxin containing 0·5 *per cent. phenol.* Chart 2.

In a preliminary experiment to see whether acid destroyed phenolised toxins, there was over 30 per cent. destruction of the Lf value in three out of four toxins acidified with 4 per cent. glacial acetic acid and tested after neutralising a week later.

In the first experiment phenol (0·5 per cent.) was added to the toxin, which was then similarly treated with acid and alkali. Chart 2 shows the same approximate limits of precipitation as chart 1 but not the same distribution of the toxin. The maximum amount of toxin precipitated is greater; out of the initial 14 units, 6·5 units were precipitated from the formalised toxin and 9·5 after the addition of phenol.

There is a second difference—the precipitates in the zone between *p*H 2·5 to 5·5 are less readily soluble, and the toxin once precipitated

is not completely recoverable. In chart 2 curve C (Lf value of the whole solutions) joins curve B (clear filtrate) at *p*H 3·2 which shows that none of this precipitated toxin went into solution on reneutralising the whole suspension. In chart 1 this occurred only below *p*H 1·0.

Formalised toxin + 2 *per cent. alum.* Chart 3.

Two per cent. alum was added to a large volume of the toxin; it lowered the *p*H to 3·9 and brought down a heavy precipitate; separate samples of this suspension were adjusted to various hydrogen ion concentrations as rapidly as possibly and left for 48 hours.

All the samples between *p*H 2·5 and 10·6 precipitated and the amount of precipitate was much less at the outer limits of this zone. At *p*H 2·0 the toxin was clear, at *p*H 0·6 it again precipitated slightly. On the alkaline side, the heavy precipitate produced by the alum tailed off into the fine precipitate found with alkali alone without any break. There was only one small clear zone round *p*H 2·0.

Neither the supernatant nor the precipitate from the toxin after the addition of 2 per cent. alum could be titrated by flocculation with antitoxin. The precipitate was not soluble in neutral solution; the supernatant was too acid to flocculate direct with antitoxin, and neutralising with alkali brought down a further precipitate. This second precipitate was largely aluminium hydroxide, but it could not be taken for granted that no toxin would be lost by filtering it off.

The aluminium ions must be removed before the Lf value can be determined; this was done with Rochelle salt. Aluminium hydroxide, alone almost insoluble in neutral solution, dissolves in the presence of neutral alkali tartrates forming a complex anion. Preliminary experiments with several toxins showed that 2 per cent. Rochelle salt itself had no effect on the Lf value; this amount was found sufficient to dissolve the precipitates and to keep the filtrates in solution on adjusting the *p*H to 8·0. The reaction is slow: the precipitates are not readily soluble and to get a clear solution both time and frequent shaking are necessary. The HCl in the more acid filtrates in experiment 3 precipitated tartaric acid when the solution of Rochelle salt was added. It was found best to add the alkali and tartrate alternately little by little.

In the clear solution: at *p*H 10·6 no Lf units were detectable; at *p*H 9·6 the value was 10; it then dropped to a minimum of less than 1 unit at *p*H 5·0 and rose as the precipitate dissolved with more acid to 9·5 units in the clear solution at *p*H 2·4. Finally when the excess acid precipitate came down at *p*H 0·8, there was no detectable toxin left in solution.

The toxin which disappeared from the clear solution in the zone *p*H 3·0 to 9·0 was recoverable when these precipitates were dissolved; as before, the excess acid and alkaline precipitates could not be got

into solution. From the fact that the Lf value falls at the first trace of precipitate in the excess acid zone, and that precipitation precedes the fall with excess alkali, it is possible that the toxin in the acid suspensions is in the insoluble precipitates, while in the alkaline suspensions it is destroyed independently of precipitation, as was shown by the antigenic results in chart 1. The original amount of toxin was recoverable in the control solutions between *p*H 6·4 and 8·4, but there was a slight increasing loss from *p*H 6·4 to 3·4.

The antigenic values of the alum fraction, precipitate and filtrate, at *p*H 4·0 were as high as that of the original toxoid. This fact was confirmed on another toxoid when an alum precipitate and its filtrate containing less than 1 Lf unit per c.c. were each, volume for volume, better antigens than the parent toxoid containing 10 Lf units per c.c. The comparison was made with varying initial doses.

Later experiments showed that 0·2 per cent. alum was enough to precipitate all the toxin when adjusted to the isoelectric point. Without the addition of any acid the minimum amount of alum to precipitate the toxin was 0·8 per cent. (*p*H 4·7); smaller amounts although giving a precipitate did not lower the *p*H sufficiently to precipitate the toxin.

Formalised toxin + 2 per cent. alum + 2 per cent. Rochelle salt. Chart 4.

A further experiment was made to precipitate a toxin in the presence of fewer aluminium ions. For this purpose 2 per cent. Rochelle salt was added to a toxin before the addition of 2 per cent. alum. It will be seen from chart 4 that the presence of the tartrate narrowed the zone of precipitation and the curve is intermediate in appearance between curves 1 and 3. The toxin is recoverable from the precipitates on adjusting the *p*H to 8·0.

Other precipitants.

0·5 per cent. uranium nitrate precipitates the Lf units from a toxin at *p*H 6·2, the precipitate is antigenic and contains about one-fourth of the original nitrogen. Hedin's tannin solution and trichloracetic acid (2 per cent.) both precipitate toxins, as measured by Lf units or antigenic value, when the *p*H falls to 4·0; and 1 per cent. ferric alum and aluminium nitrate have also been used successfully.

Conclusions.

1. Three distinct zones of precipitation are produced by the action of acid or alkali upon a toxoid containing 2 per cent. formaldehyde.

   (*a*) At, and below *p*H 1·0 all toxin, as measured in Lf units, disappears from solution and the precipitate does not dissolve on the addition of alkali. Toxin is not destroyed as low as *p*H 0·2 since an emulsion of the precipitate separated at that level has high antigenic value.

(*b*) Between *p*H 2·5 and 5·5 at the point of optimum precipitation 60 per cent. of toxin remained in solution, and 40 per cent. was precipitated and could be recovered in solution by the addition of alkali.

(*c*) Above *p*H 8·6, although a precipitate is formed, toxin remains in solution until a *p*H of 10 is reached. Between 10 and 11 toxin disappears from solution and cannot be recovered from the precipitate. Since an emulsion of the precipitate is only feebly antigenic the toxin must have been destroyed by alkali.

2. The presence of 0·5 per cent. phenol increases the maximum amount of toxin precipitable between *p*H 2·5 and 5·5 to 70 per cent. The precipitate is less soluble and loss of Lf units occurs unless the precipitate is handled rapidly.

3. With the addition of 2 per cent. alum the zone of *p*H within which toxin is precipitated is much wider than with acid alone and practically no toxin is left in solution between *p*H 3·8 and *p*H 6. Although the yield of precipitated toxin is greater the purification is less, but the antigenic value of the toxin is greatly increased by the presence of alum. The minimum concentration of alum that lowers the *p*H to 5·0 will precipitate the toxin. Smaller amounts of alum will also do so if the *p*H is independently lowered with acid to the isoelectric point. Toxin precipitated with alum is soluble on the addition of Rochelle salt and soda to raise the *p*H to 8·0.

4. Ferric alum, aluminium nitrate, uranium nitrate, 2 per cent. trichloracetic acid and 2 per cent. Hedin solution will precipitate toxin.

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REFERENCES.

Brieger and Boer, 1896 . .	*Deutsche med. Woch.*, xxii. 783
Brieger and Frankel, 1890 .	*Berlin klin. Woch.*, p. 241.
Doerr, 1908	*Biochem. Zeit.*, vii. 128.
Glenny, Allen and Hopkins, 1923	*British Journ. Exper. Path.*, iv. 19.
Glenny, Pope, Waddington and Wallace, 1926	this *Journal*, xxix. 38.
Glenny and Wallace, 1925 .	*ibid.*, xxviii. 317.
Glenny and Walpole, 1915 .	*Biochem. Journ.*, ix. 298.
Hedin, 1903	*Journ. Physiol.*, xxx. 156*n*.
Moloney and Weld, 1925 . .	*Studies from the Connaught Laboratories, University of Toronto*, p. 263.
Pope and Gowlett	*Journal of Scientific Instruments* (in press).
Walbum, 1922	*Biochem. Zeit.*, cxxx. 25.
Watson and Wallace, 1924 .	this *Journal* (*a*) xxvii. 271, (*b*) xxvii. 289.

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